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Separation of Proteins by Gradient Solvent Extraction of a Protein Precipitate

Charles A. Zittle and Edward S. DellaMonica

From the Eastern Regional Research Laboratory,[1] Philadelphia, Pennsylvania

Introduction

A method for the separation of proteins has been described by Zahn and Stahl (1) in which a precipitate of the proteins is extracted with a continuously diluted solvent. Specifically, a mixture of proteins was precipitated with ammonium sulfate and the precipitate was extracted with ammonium sulfate solution continuously diluted with water. The method was applied by Zahn and Stahl (1) to a yeast extract containing several enzymes. The present report confirms the usefulness of the method with a mixture of known proteins, namely bovine serum albumin and human hemoglobin. The method has also revealed changes in the solubility of α-lactalbumin that had been dried from the frozen state and stored at 25°.

Proteins Used and Their Determination

The bovine serum albumin was a crystalline commercial product. It was precipitated once before use with 3.0 *M* ammonium sulfate at pH 8.5, and freed of salt by dialysis.

The human hemoglobin was obtained by hemolyzing red blood cells with 6 vol. of 0.01 *M* acetic acid, and removing the cell membranes by sedimentation. The hemoglobin was precipitated with ammonium sulfate in the same manner as the albumin. This was homogeneous on electrophoresis.

The α-lactalbumins were crystalline materials prepared by Dr. W. G. Gordon (2). One of the preparations had been dried from the frozen state and stored at 25°, with 9% moisture content, for 2 years.

The concentrations of the proteins in these experiments were calculated with the use of light-absorption coefficients determined on solutions of the purified pro-

[1] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

teins. Albumin: 1.0 mg. albumin/ml. gives an absorbance of 0.63 at 280 mμ in a 1-cm. cell. Hemoglobin: 1.0 mg. hemoglobin/ml. gives an absorbance of 7.5 at 415 mμ. The absorption of hemoglobin at 280 mμ is 0.30 of the absorption at 415 mμ. The determination of albumin in mixtures of the two proteins is based on total absorption at 280 mμ less that due to hemoglobin. α-Lactalbumin: 1.0 mg. α-lactalbumin/ml. gives an absorbance of 1.80 at 280 mμ.

Equipment for Diluting Solvent; Calculation of Concentration

The continuously diluted solvent is obtained with equipment similar to that described by Zahn and Stahl (1) but uses standard glassware with some modification. The mixing vessel which also serves as a reservoir, is an open top, Pyrex separatory funnel, size 125 ml., to which a side arm has been added. Water, or other solvent, is fed into the side arm of the mixing vessel from an inverted bottle. The feed tube has an inside diameter of 7 mm., and the end is cut on a slant so that capillarity does not prevent the free flow of liquid. The solvent coming from the mixing vessel enters a Pyrex-glass Büchner funnel 30-ml. size with a coarse fritted-glass plate which contains the protein precipitate mixed with an inert carrier (Hyflo SuperCel is satisfactory).[2] The solvent is kept stirred in the mixing vessel and is permitted to flow through the system at 5–10 ml./10 min.

The concentration of salt in any sample[3] is a function of the volume (v) and the numer (n) of the sample, the volume (V) and concentration (C_0) of the solution in the mixing vessel, and the concentration of the diluent in the inverted bottle. When the bottle contains water, the decrease in concentration in the mixing vessel is described by $(v/V).C_0.dn$. On integration, log (C/C_0) equals $-v.n/V.2.30$. When the bottle (B) contains a dilute salt solution, $C_0 - C_B$ can be used in place of C_0, and the true value of C will be the calculated C plus C_B. The experimental dilution curves, determined for each 5 ml. of effluent with the Nessler reagent, have been in agreement with calculated values as shown in Fig. 1. The experimental samples at the beginning showing no change in concentration represent the precipitating solution (20–30 ml.) and the solution displaced from the side arm of the reservoir (about 20 ml.) by the diluting solvent.

Procedure

The application of the solvent-gradient method to a specific mixture of proteins is shown by the following. A mixture of albumin (100 mg.) and hemoglobin (50 mg.) in a volume of 5.0 ml. is precipitated by the addition of 15.0 ml. of 4.0 M ammonium sulfate adjusted to pH 8.5 (prepared by the addition of 5.0 ml. of ethanolamine to 750 ml. of saturated ammonium sulfate; the pH was measured undiluted). Seven grams of Hyflo SuperCel is added to the mixture of precipitated

[2] Mention of products does not imply endorsement or recommendation by the U. S. Department of Agriculture over other products of a similar nature not mentioned.

[3] We are indebted to C. Ricciuti of this laboratory for discussion of the formulation which follows.

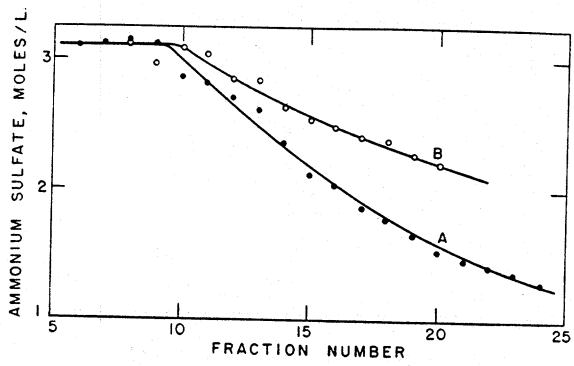

FIG. 1. Relation of ammonium sulfate concentration to fraction number. Both curves were obtained by the dilution of 100 ml. of 3.1 *M* ammonium sulfate, and the collection and analysis of 5.0-ml. fractions. Curve *A* was obtained by dilution with water, curve *B* by dilution with 1.2 *M* ammonium sulfate. The points represent experimental data; the lines are calculated from the equation given in the text.

proteins. Before this is placed in the Büchner, 0.5 g. of Hyflo is suspended in several milliliters of solvent and placed on the plate in the Büchner. Excess solvent is forced out by pressure, and the mixture of proteins and Hyflo is introduced. Pressure is exerted until the top of the column is just freed of liquid. Precautions are taken that the top of the Hyflo column is level. The space above the Hyflo is filled with the solution used for precipitating the proteins, and a stopper, which contains two glass tubes, is introduced. The center tube is connected with the mixing vessel. The second tube in the stopper remains open until the air space is filled with liquid. The 100 ml. of 3.1 *M* ammonium sulfate placed in the mixing reservoir is gradually diluted with 1.2 *M* ammonium sulfate, and the mixed diluted solvent is passed through the Büchner. Successive 5-ml. samples are collected and analyzed.

RESULTS AND DISCUSSION

The results obtained with the mixture of albumin and hemoglobin are given in Fig. 2. The albumin appears in the solvent effluent first, followed shortly by the hemoglobin. The concentration of the solvent in each sample number is given by curve *B* of Fig. 1. The concentration gradient illustrated by curve *A*, Fig. 1, for which 100 ml. of 3.1 *M* ammonium sulfate was diluted with water, changed too rapidly to permit the separation of albumin and hemoglobin. The concentration of the solvent which brings each protein into solution corresponds closely to that expected from individual precipitation experiments. For example, when a 4.0 *M* solution of ammonium sulfate is added to 5.0 ml. of a 2.0 % solution

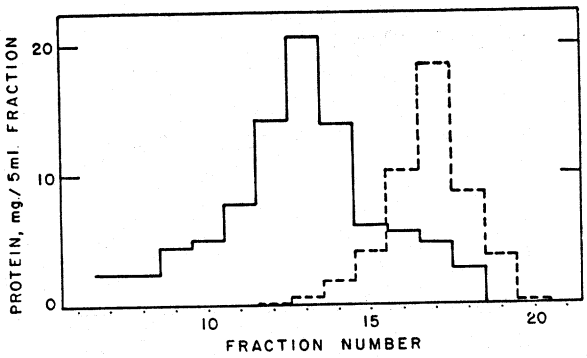

FIG. 2. Separation of serum albumin and hemoglobin by gradient extraction of the ammonium sulfate precipitate of the mixed proteins. The proteins were precipitated with a 3.1 *M* concentration of ammonium sulfate. The precipitate was extracted with continuously diluted ammonium sulfate (concentrations are given by curve *B* of fig. 1). The solid line represents the albumin in 5.0 ml. of extract; the dashed line represents the hemoglobin.

of albumin, the solution remains clear at 2.68 *M* ammonium sulfate, and a heavy precipitate is obtained when the concentration reaches 2.84 *M*. In several experiments similar to that shown in Fig. 2, the bulk of the albumin is extracted by 2.75 *M* ammonium sulfate. In a similar experiment, a solution of hemoglobin remained clear at 2.32 *M* ammonium sulfate, and some precipitate is obtained with a concentration of 2.48 *M*. In the above serial extraction experiments, the bulk of the hemoglobin is extracted at a concentration of 2.5 *M* ammonium sulfate.

The solubility of α-lactalbumin was studied with the ammonium sulfate concentration and other conditions the same as in the albumin–hemoglobin experiments. In this instance when the 2.0 *M* ammonium sulfate had been diluted with 100 ml. of 1.2 *M* ammonium sulfate, the dilution was continued with water. Under these conditions the change in concentration of ammonium sulfate was approximately linear throughout the experiment, decreasing about 0.8 *M* for each 50 ml. of extractant.

A 150-mg. portion of crystalline α-lactalbumin that had been dried from the frozen state and stored at 25° gave the results shown by the solid line in Fig. 3. The most soluble component, which constituted about 20% of the total, was brought into solution by a 2.5 *M* concentration of ammonium sulfate (fraction No. 16) whereas the less soluble component did not dissolve until the concentration had dropped to 1.5 *M* (fraction No. 32). Since the purity of this protein had been fully

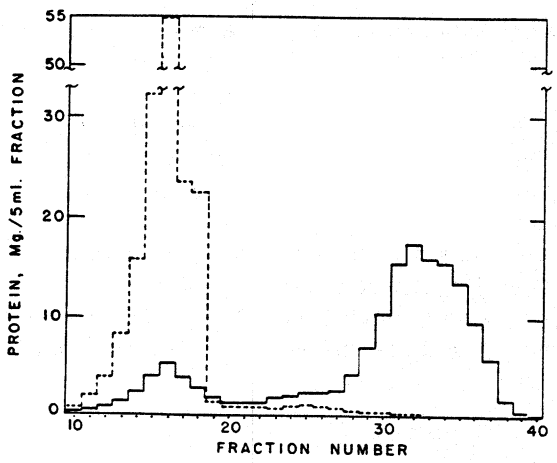

FIG. 3. Gradient extraction of ammonium sulfate precipitates of α-lactalbumin. Crystalline α-lactalbumin dried from the frozen state, solid line; an undried preparation, dashed line.

established before drying, it was suspected that the less soluble component represented α-lactalbumin that had altered on storage. An experiment with 150 mg. of α-lactalbumin that had not been dried and had been stored at 7° gave the dashed line of Fig. 3. This preparation is almost entirely the component of higher solubility, with a few per cent of a less soluble component. The ease with which α-lactalbumin changed to a less soluble protein is unusual, especially in view of the low molecular weight [15,100 (2)] of this protein. This change is not the usual denaturation, for when ammonium sulfate solutions of the less soluble component (for example, sample No. 32 of the experiment illustrated in Fig. 3) are heated in a 100° bath, a precipitate forms. Presumably this precipitated material, of even less solubility, represents the fully denatured α-lactalbumin.

The advantages for protein fractionation of extracting a precipitate of proteins with a suitable solvent compared with separation by precipitation have been pointed out by Cohn *et al.* (3). Some of the advantages are the greater stability of proteins in the precipitated state and the more rapid attainment of equilibrium in extraction than in precipitation. To these advantages the use of a solvent gradient adds increased ability to differentiate the proteins in a mixture. If there is no interaction among the proteins, each will be extracted in turn as a suitable concentration of solvent is attained. Interaction among proteins does occur, but it is